

CHAPTER 2

Sample Collection and Submission

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I. Introduction

The purpose of this section is to provide guidance to laboratory staff and user groups regarding the proper sample collection and submission procedures. This is an absolute necessity to assure that samples received by the Centers are acceptable specimens for definitive pathogen identification. Each sample submission must include a NWFHS Submission Form (Appendix B). Specimens will be stored, maintained and processed in accordance with protocols described in subsequent chapters of this Manual. Sampling procedures not found in this Manual will follow diagnostic procedures outlined in *Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens*, 1994, 4th edition. American Fisheries Society, Fish Health Section, Bethesda, Maryland (AFS Blue Book).

Additional, detailed information regarding sample collection and processing can be found for specific assays in the following chapters:

- Chapter 3 - Sample Receipt and Laboratory Tracking
- Chapter 4 - Standard Necropsy Procedures for Finfish
- Chapter 5 - Bacteriology (*Aeromonas salmonicida*, *Yersinia ruckeri*, *Edwardsiella ictaluri*)
- Chapter 6 - ELISA for Detection of *Renibacterium salmoninarum* Antigen in Fish Tissue
- Chapter 7 - Corroboration Testing of *Renibacterium salmoninarum* by PCR
- Chapter 8 - Parasitology (*Myxobolus cerebralis*, *Bothriocephalus acheilognathi*, *Ceratomyxa shasta*)
- Chapter 9 - Corroboration Testing for Parasites by PCR
- Chapter 10- Tissue Culture
- Chapter 11- Virology
- Chapter 12- Corroborative Testing of Viral Isolates
- Chapter 13- Histology
- Chapter 14- Non-Lethal Methodology for Detection of Fish Pathogens

II. Considerations for Sampling Fish Populations

Disease Recognition and Action – The majority of sampling conducted under the Survey will occur when no external signs of disease exist in wild fish populations. However, the Survey may also be helpful in determining the cause of fish kills or monitoring wild populations when abnormal behavior patterns, external abnormalities, or high mortality are reported for natural fish populations. In these cases, an immediate response is needed to determine the cause of mortality and determine if infectious agents are present or if adverse environmental conditions exist (low dissolved oxygen, elevated temperatures, toxic algal bloom, water contaminants, etc.). The following offers guidelines for sampling fish under various scenarios:

- In clinical cases of disease ($\geq 0.5\%$ mortality/day) 10 moribund fish are generally sufficient to detect fish pathogens and make a disease diagnosis.

- In survey or monitoring situations where no excessive mortality or clinical disease is apparent, a larger sample size of 60 animals may be necessary to detect infection rates below 5%. However, depending upon individual circumstances, sample sizes may vary between 10 and 60 fish. Samples should be examined from several stretches of a stream or body of water to ensure representation of the entire population. It is also extremely important that sampling techniques are optimal and laboratory assays as sensitive as possible to allow detection of fish pathogens, especially when sample numbers are small (<60 fish).
- Donor populations – in some cases, natural populations will be used as donor broodstock to provide gametes to a hatchery program involved with a captive broodstock program or restoration activities. When a disease history for natural population is needed, a minimum of 60 samples from spawning adults is required to detect pathogens at a 5% prevalence and a confidence interval of 95%. Samples of choice are from spawning or post spawning individual female fish consisting of ovarian fluid and kidney/spleen tissue.

The priority for acceptable samples submitted for Wild Fish Survey testing is as follows:

- Live specimens or samples taken on site
- Iced specimens or tissues
- Preserved specimens or tissues

III. Sample Collection

Prior to collecting samples, the Center will contact sampling personnel with instructions on the appropriate types of samples and numbers of fish needed. Partners collecting specimens need to provide at least one-week advance notice of sample collection. If advance notice is not given, lab personnel may not be available to receive and process the submission after it is collected and shipped. Samples that are not in an acceptable condition (either substandard or improperly packaged) upon arrival cannot be processed due to the poor sample quality and unreliability of pathogen testing.

The following instructions are general guidelines. User groups and or individual collectors should be properly trained in the use of these sampling procedures by a fish health specialist, pathologist, or technician prior to sampling fish. Different procedures are followed in bacteriology, virology, parasitology, ELISA, PCR, and histological analyses.

Further details regarding the procedures below are addressed in the appropriate chapters of this Manual.

- Live fish are preferred, and should be sampled immediately upon removal from the water. If this is not possible, fish should be held on ice and processed within 1-2 hours after collection. Whole fish can be packaged live, or freshly killed (iced) for shipment to the

Centers for processing within 24 hours. When individual tissues are collected, these should be kept cold with ice or artificial icepacks but should not be allowed to freeze (insulated from direct contact with ice).

- Samples for virology should be processed within 48 hours and inoculated into cell culture within 72 hours of sample collection. Upon specimen arrival determine the freshness of the fish. Criteria for this freshness are: smell, appearance of eyes, gills, internal organs and presence of postmortem stiffness (rigor mortis). If shipping is delayed beyond 24 hours, some tissues may not be suitable for processing.
- Euthanize the fish or group of fish. Use clean sterile dissecting instruments. Clean dissecting tools with soap and water and disinfect with alcohol between the sampling of each individual fish (species) or species group. The use of disposable tools is recommended for ELISA samples, as Rs antigen is difficult to remove by standard disinfection methods. Disinfect hands between the sampling of each fish (species) or species group. Before taking tissue samples, wipe clean any mucus or debris from the fish and then disinfect the outer surface of the fish by flooding with 70% alcohol. Proceed with necropsy of fish tissues.

IV. Bacteriology

Bacteriological samples should be taken first to reduce the chance of contamination. If open sores or lesions are present swab these areas and streak the sample onto a BHIA agar slant. Discard swab and re-cap slant. The abdominal cavity is entered by cutting into the abdominal wall at the base of the pectoral fin with a pair of small sterile scissors or scalpel. The cut is continued dorsally to just below the lateral line. Start again at the base of the pectoral fin and continue the incision towards the posterior of the fish along the ventral abdominal wall to the vent. Stay slightly above the intestinal tract when making the incision so that it is not punctured, thereby contaminating the abdominal cavity and target tissues.

Use the butt end of a sterile inoculating loop to pull back the internal viscera and air bladder to expose the kidney. Stab the kidney with a sterile inoculating loop and streak on to a BHIA agar slant. Discard the loop and re-cap the slant. Record sample number and date on tube. Sample numbers can be any logical group of letters and numbers in order (RBT-1, RBT-2). Store tubes in a cool place such as cooler or cool room (15-20°C).

V. Virology

Kidney/Spleen: Dissect approximately 0.5 gm piece of kidney and/or spleen and place into a small WHIRL-PAK™ bag or snap-cap tube with a small amount of HBSS (Hank's Balance Salt Solution) to cover sample. Keep the HBSS cold at all times. Label bag with sample number, K/S and date. Up to 5 fish samples may be pooled in one bag or tube if appropriate. Keep samples on ice while in the field. Samples must be kept cold (5-18°C), but **do not freeze**, during

shipment to the Center.

Ovarian Fluid: Sexually mature females only. Remove a small amount of ovarian fluid from the oviduct using a pipettor or express ovarian fluid into a paper cup (approximately 1 ml per fish) Place the fluid in a small snap-cap or transport tube. Up to five (5) fish may be pooled into one sample. Label tube with sample number (OV-1, OV-2, etc.) and date. Keep samples cold (4°C) with pre-packaged ice packs, but **do not freeze** during collection or transport. Sample tubes should be placed within a WHIRL-PAK™ or zip- lock bag which is labeled with the stream or lake name, fish species, sample type, etc.

VI. ELISA for *Renibacterium salmoninarum* (BKD)

Remove the remaining amount of kidney or sizable portion using **individual**, or **disposable** instruments. Place the kidney tissue into a snap-cap tube or small WHIRL-PAK™ bag. Up to fish five can be pooled into one sample if necessary to obtain a minimum tissue weight of 0.08g per sample (0.08g diluted 1:8 during processing will yield 560ul for the ELISA assay). Label with sample number, KD-ELISA and date (similar numbering scheme as with bacteriology slants). Keep samples on ice for transport to the lab. Samples can be frozen at -20°C for delayed processing, however if long-term storage is needed, -70°C is recommended.

VII. Parasitology

Myxobolus cerebralis (Whirling Disease):

Salmonid Fishes Only. For small fish remove the entire head and gill arches. For larger fish, take a cranial core sample and/or gill arches. If taking a core sample, see Chapter 9 – Corroborative Testing of Parasites by PCR, for the target tissue site for *Myxobolus cerebralis*. Up to (5) fish may be pooled into one sample. Place samples in a WHIRL-PAK™ or zip-lock bag labeled with sample information. Pack samples on ice for transport to the CENTER.

Either during sample collection, or during processing in the laboratory, the head tissue is halved into two separate pieces to allow testing of one half by Pepsin-Trypsin Digest (PTD) and archiving of the second specimen for corroborative testing by PCR or histology. If tissue is halved during collection, label each half to allow correlation between archive and tissue to be used for PTD tissues. Recommend a notation of “PTD” and “ARC”, as well as FISH ID NUMBER on each 5-pool sample (e.g., PTD 1-5, PTD 6-10, ARC1-5, ARC 6-10, etc). Tracking the fish identification for each sample pool will facilitate corroborative testing by PCR. In this way, only the PTD positive sample pool will require corroborative testing of the archive sample by PCR, rather than the entire sample set.

Bothriocephalus acheilognathi (Asian Tapeworm) and *Ceratomyxa shasta* (salmonid ceratomyxosis):

Remove the GI track of the fish from the esophagus to the anus. Place GI track into WHIRL-PAK™ or zip-lock bags. Small fish of the same species can be pooled (5 fish) if applicable. Label samples with appropriate information. Pack samples on ice for transport to the Center.

VIII. Histological Samples

Histological samples should be fixed in Bouin's solution, 10% buffered formalin, Davidson's fixative, or Prefer fixative. Fix live fish after anesthetizing. Fix tissues within **2 minutes** of removal from water and/or time of death. Fish rapidly autolyze (especially gill) and only freshly fixed tissues are worth processing for histological analysis. The volume of fixative must be at least 10 times the volume of tissue. For fish longer than 6 cm, slit the abdomen, detach the intestine at the anus, and pull the internal organ mass out slightly to allow penetration of fixative within the body cavity. For larger fish, send only specified organs in fixative. Cut tissues with a sharp blade or scissors - don't tear as this action creates artifacts.

SAFETY NOTE ! formalin-based fixatives are toxic and strong irritants.

Read the entire Material Safety Data Sheet. Avoid contact with skin and eyes by wearing gloves and a face shield. Use only in well-ventilated areas (outdoors or under a fume hood).

HAZARDOUS MATERIALS - Fixatives and alcohol require special shipping procedures as Dangerous Goods, or Hazardous Materials. Refer to your local regulatory agency and commercial carrier for requirements for shipping these materials.

Place a paper **penciled** label (location, date, species, tissue type, and initials) **inside** the fixative container (alcohol and fixatives tend to wash off pen marks on the container).

Prevent spills during transport. Tightly cap the container, then wrap the cap with several layers of parafilm, and place the containers inside Ziploc bag(s). If samples fixed in Bouin's or Davidson's Fixative cannot reach the laboratory in 48 hrs, it will be necessary to transfer fixed tissues to 70% ethanol after 24-48 hours and then transport the tissues to the Center (Samples fixed in 10% Buffered Formalin or Prefer fixative can be held in fixative indefinitely).

IX. Non-lethal Collection of Tissue Samples

Compliance with the Endangered Species Act (ESA) of 1973 requires special consideration regarding take of threatened or endangered (T&E) species if they occur in a proposed sampling site or watershed. All Centers and Partners in the Survey are responsible for obtaining

appropriate collection permits, coordinating sample collection with Federal, State and local regulatory agencies, and fully complying with the regulatory statutes of the Endangered Species Act.

When lethal sampling of T&E species is prohibited, non-lethal sampling techniques should be considered. While non-lethal sampling methods are less sensitive than standard detection methods, they may provide limited fish health information when no other alternative exists. Refer to Chapter 14 -Non Lethal Methodology for Detection of Fish Pathogens for specific protocols for non-lethal sampling.

Bacteriology

Blood Samples

Obtain blood via heart puncture or caudal vein or artery using a needle and syringe. Streak blood onto BHIA slant with sterile loop. Discharge appropriate amount of blood directly into the appropriate volume of PBS-T20 for ELISA. Heparinized blood may be used for delayed transport and processing.

Ovarian Fluid Samples

If the fish is a sexually mature female, remove a small amount of ovarian fluid and inoculate a BHIA plate for bacterial growth. Also, the same sample can be placed into a centrifuge tube. Up to five (5) fish may be pooled into one sample. Keep samples cold for transport, but **do not freeze**. Following centrifugation and processing for virology, the Ovarian Fluid Pellet can be used to screen for *Renibacterium salmoninarum* by FAT.

Mucus Samples

Pass a sterile swab along the lateral surface of the fish. Streak the sample onto a BHIA agar slant.

Vent Samples

Place a sterile swab or loop approximately ½ to 1 inch into the anal vent and remove. Streak the sample onto a BHIA agar slant. (Discard the swab or loop).

External Lesions

Pass a sterile swab along the surface of an external lesion. Streak the sample on selective media for the targeted bacteria. Plate serial dilutions of the inoculum to decrease the number of interfering bacteria and fungi that are likely to be present in this type of sample.

Virology

Blood Samples

Obtain blood via heart puncture or caudal vein or artery using a needle and syringe. Discharge appropriate amount of blood directly into the appropriate volume of antibiotic-antimycotic incubation (anti-inc) solution. Keep samples cold for transport, but **do not freeze**. Follow normal processing protocol.

Ovarian Fluid Samples

If the fish is a sexually mature female, remove a small amount of ovarian fluid and place into a centrifuge tube. Up to five (5) fish may be pooled into one sample. Keep samples cold for transport, but **do not freeze**. Follow normal processing protocol.

Mucus Samples

Collect mucus by passing a blunt edge instrument along the lateral surface of the fish, head to tail. This is easily accomplished when the fish is removed from the water, holding the fish head to tail in a vertical position. Mucus is placed in a 15 (ml) or smaller graduated centrifuge tube with antibiotics used in viral sample processing. Keep the samples cold for transport to the Center, but **do not freeze**. In the lab, samples should be vortexed, and then a low dilution scheme (1:2, 1:5, 1:10) is set up using Hanks Balanced Salt Solution (HBSS). Samples are centrifuges at low speed, 2000-3000 rpm for ten to pellet cellular debris. Supernatant is inoculated into cell culture.

Fecal samples

Feces are collected by aspiration with a syringe and small tubing catheter. Samples are placed into small collection tubes with antibiotics (may need to increase concentrations of antibiotics by 25-50% for fecal samples). Keep the samples cold for transport, but **do not freeze**. Follow processing as in mucus samples (may require higher dilutions to avoid toxicity to cell lines).

External Lesions

Pass a sterile swab along the surface of an external lesion. Place the swab in anti-inc solution pressing, or rolling the swab against the interior of the tube to release the material into solution. Keep samples cold for transport, but **do not freeze**. Follow normal processing protocol.

Parasites

Blood Samples

Obtain blood via heart puncture or caudal vein or artery using a needle and syringe. Discharge appropriate amount of blood directly into a slide and prepare a thin blood film (Chapter 4, page 11). Blood films are air dried, and fixed in absolute methanol for 10 minutes. Blood parasites can be viewed following staining with Diff-Quick or a Gram stain (Chapter 4, page 10).

Mucus Samples

Collect mucus by passing a blunt edge instrument along the lateral surface of the fish, head to tail. This is easily accomplished when the fish is removed from the water, holding the fish head to tail in a vertical position. Mucus is placed in a 15 (ml) or smaller graduated centrifuge tube. The tissue can be examined directly under microscopy for parasites or kept cold for transport and examined in the laboratory.

Fecal samples

Feces are collected by aspiration with a syringe and small tubing catheter. Samples are placed into small collection tubes. The tissue can be examined directly under microscopy for parasites or kept cold for transport and examined in the laboratory.

External Lesions

Pass a sterile swab along the surface of an external lesion. Place the swab in normal saline or PBS solution pressing, or rolling the swab against the interior of the tube to release the material into solution. The tissue can be examined directly under microscopy for parasites or kept cold for transport and examined in the laboratory.

Tissue Biopsy

Gill filament can be removed from anesthetized fish with little injury to the fish. The tissue can be examined directly under microscopy for parasites, preserved for histology, or frozen for examination using other diagnostic methods. See Chapter 14 for more a detailed protocol for gill biopsy.

Water and sediments sampling for virus, bacteria and parasites is also discussed in Chapter 14 - Non-Lethal Methodology for Fish Pathogens.

X. Shipping Samples – See Appendix A for shipping addresses of Centers

- A. Ship samples in small/medium (heavy duty) mailing cartons or plastic coolers lined with a plastic trash bag. Group the samples by type in separate Ziploc bags or racks that are labeled with the number of samples, location, species, and date. Position the samples upright and use packing material to hold samples in place.
- B. Place an adequate amount of ice (or artificial gel packs) around the insulating layer of packing material. Seal the outer trash bag to prevent leakage.
- C. Complete the NWFHS Submission Form (Appendix B) for each species and enclose in a waterproof plastic bag within the cooler.
- D. Close, seal and label the ice chest with laboratory address, be sure to include the Center contact's NAME AND PHONE NUMBER).

- E. Appropriate shipping labels should also be affixed to ensure proper handling during shipment and upon receipt of containers.

“Live Fish – Do Not Freeze”for live samples
“Keep Frozen”for ELISA and/or Head tissue
“Refrigerate but DO NOT FREEZE”for virology or sets of samples that
include both Bacteriology and Virology
samples.

NOTE: Temperature indicator strips can be placed with the samples.
These strips will change color if 20°C has been exceeded during shipment.

- F. Transport within 24 hours via overnight U.S. mail or Federal Express.

NOTE: Collectors should always include a Submission Form with samples to provide the collection information for the laboratory tracking and database entry. Samples submitted without this information may be refused or at least delayed for processing. When in doubt regarding collection and shipping instructions, consult the Center contact for the Survey.

Centers are prepared to provide all supplies needed for field sampling and training in sample collection. Transportation costs, personnel to assist with sampling and shipping costs may be provided if possible.

XI. References

Amos, K.H., editor. 1985. Procedures for the detection and identification of certain fish pathogens. 3rd edition. Fish Health Section, American Fisheries Society, Corvallis, Oregon. 114 pp.

CA-NV FHC, 1997. Histological sampling of fish tissues. S.O.P., California-Nevada Fish Health Center. Anderson, CA. 2 pp.

Lasee, B.A., editor. 1995. Introduction to fish health management. 2nd edition. U.S. Fish and Wildlife Service, La Crosse Fish Health Center. Onalaska, WI. 139 pp.

Meyers, Theodore R., editor 1997. Fish pathology section laboratory manual, special publication No. 12, Alaska Department of Fish and Game.

Mitchell, A.J., and G.L. Hoffman. Submitting samples for fish disease diagnosis. U.S. Fish and Wildlife Service, Fish Farming Experimental Station, Stuttgart, AR. 15 pp.

Plumb, J.A., and P.R. Bowser. 1983. Microbial fish disease laboratory manual. Alabama Agricultural Experimental Station, Auburn University, AL. 95 pp.

Thoesen, J.C., (ed). 1994. Suggested procedures for the detection and identification of certain finfish and shellfish pathogens (4th Edition). Fish Health Section. American Fisheries Society, Bethesda, MD.

Appendix A - Shipping Addresses and Contacts for Fish Health Centers

Bozeman Fish Health Center
920 Technology Blvd, Suite G
Bozeman, MT 59718

Crystal Hudson
Kenneth Peters
406-582-8656
406-587-3998 (fax)

California-Nevada Fish Health Center
24411 Coleman Hatchery Road
Anderson, CA 96007

Scott Foott
Kimberly True
530-365-4271
530-365-7150 (fax)

Idaho Fish Health Center
P.O. Box 272
Orofino, ID 83520
FOR SHIPPING:
4447 Ahsahka
Ahsahka, ID 83520

Kathy Clemens
Laura Kessel
208-476-9500
208-476-9741 (fax)

La Crosse Fish Health Center
555 Lester Avenue, Suite 100
Onalaska, WI 54650-8552

Rick Nelson
Becky Lasee

608-783-8444
608-783-8450 (fax)

Lamar Fish Health Center
P.O. Box 155
Lamar, PA 16848

FOR SHIPPING:
Hatchery Road
Lamar, PA 16848

John Coll
Patricia Barbash

570-726-6611
570-726-7379 (fax)

Lower Columbia Fish Health Center
61552 SR14
Underwood, WA 98651

Susan Gutenberger
Ken Lujan

509-493-3156
509-493-2748 (fax)

Olympia Fish Health Center
3704 Griffin Lane, Suite 101
Olympia, WA 98501

Ray Brunson
Chris Patterson

360-753-9046
360-753-9403 (fax)

Pinetop Fish Health Center
P.O. Box 160
Pinetop, AZ 85935

FOR SHIPPING:
1684 East White Mountain Blvd., Suite 7
Pinetop, AZ 85935

John C. Thoesen
Phil Hines

520-367-1902
520-367-1957 (fax)

Warm Springs Fish Health Center
5308 Spring Street
Warm Springs, GA 31830

Norm P. Heil
Robert Bakal

706-655-3382 Ext.233
706-655-3389 (fax)

Appendix B - NWFHS Submission Forms

Form 1 is a simplified version for submission of laboratory samples only.

Form 2 includes additional environmental data for the collection site.

National Wild Fish Health Survey - Submission Form 1

Submitter:		Collection Date:	
		Collection Time:	
Location:		GIS Coordinates: deg. min. sec.	
State:		Latitude:	
County:		Longitude:	
Site Description -Name of Water Body :			
Capture Method/Procedure:			
Type of samples: Whole fish Tissue(s) Slant(s) Head(s) (circle all that apply)		Number of Samples - Fish:	Species:
Pooled tissue(s) GI			
Remarks:			
Submitter Signature:		Date:	
Lab Use Only			
Lab I D:		Date Received:	
Remarks:		Time:	
		Received By:	
		Case Tracking Number:	
Case Coordinator/inspector/pathologist Signature:		Date Finished:	

National Wild Fish Health Survey - SUBMISSION FORM 2

Information in bold is mandatory

Case # _____

Submitter: _____ (partnership)		Collection Date: _____ Collection Time: _____	
Water body: _____ Reach: _____		GIS Coordinates:	
County: _____		Latitude: _____ Deg _____ Min _____ Sec Longitude: _____ Deg _____ Min _____ Sec map: T. _____ R. _____ S. _____ State: _____ Ecoregion: _____ HUC _____ 1/4 of the _____ 1/4 of the _____ 1/4	
Capture Method:		Ambient temp: _____ C	
boat e-fish weir bp e-fish hook other: _____		Water temp: _____ C	
Site Description or Common Name:		Flow: _____ cfs	
Type of Sample: _____ (circle one)		Conductivity: _____ uS/cm	
Random Selective		Turbidity: _____ ntu	
Additional Information/Comments: (including activities that affect reach. Circle all that apply)		pH: _____	
Agriculture Wilderness Roads Forestry Grazing Recreation Mining Beaver Complex Urbanization		D.O. _____ mg/L	
Submitter Signature and Date: _____		Map elevation: _____	
Number of Samples submitted: _____ fish		Rosgen stream type: _____	
(details on back)		Stream order: _____	
Ship Date: _____		Total reach length: _____	
LAB USE ONLY		Gen wetted area: _____	
Received by: (if different from submitter) _____		Stream gradient: _____	
Date: _____		Streambank condition: _____	
Time: _____		left: _____	
Tests Performed: _____ (Circle any Positives)		right: _____	
Corroborative Testing: _____		Longitudinal Habitat distribution:	
Bacteriology Y N Asal Yruck Etar Eict Fcol Fpsy _____		riffle: _____	
Virology Y N IHN IPNV OMV VHS WSIV LMBV _____		run: _____	
Parasitology Y N AT Csha Mcer _____		glide: _____	
ELISA Y N Rsal _____		pool: _____	
Other: _____		# positive pools _____ # pools confirmed _____	
Date of Completion: _____		Proper Agency Notification: _____	
Proper Agency Notification: _____		Contact Person: _____	
Contact Person: _____		Date: _____	
Date: _____		Signature: _____	
Signature: _____			

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National Wild Fish Health Survey - SUBMISSION FORM 2 - PAGE 2

Case Number:

	Genus / spp	Age A/J	Size (mm)		Sex	Whole Fish # of fish	Samp #	Fish per Pool	Pooled Specimens					ELISA # of samples Kidneys	LAB USE	
			Min	Max					Heads	Guts	Viral	Cultures	media		Rec	Init
1																
2																
3																
4																
5																
6																
7																
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